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In vivo evidence of a functional association between immune cells in blood and brain in healthy human subjects.

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1 ***In vivo* evidence of a functional association between immune cells in blood**

2 **and brain in healthy human subjects**

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17

Abstract

Microglia, the resident macrophages in the central nervous system, are thought to be maintained by a local self-renewal mechanism. Although preclinical and *in vitro* studies have suggested that the brain may contain immune cells also from peripheral origin, the functional association between immune cells in the periphery and brain at physiological conditions is poorly understood.

We examined 32 healthy individuals using positron emission tomography (PET) and [^{11}C]PBR28, a radioligand for the 18-kDa translocator protein (TSPO) which is expressed both in brain microglia and blood immune cells. In 26 individuals, two measurements were performed with varying time intervals. In a subgroup of 19 individuals, of which 12 had repeat examinations, leukocyte numbers in blood was measured on each day of PET measurements. All individuals were genotyped for TSPO polymorphism and categorized as high, middle, and low affinity binders. We assessed TSPO binding expressed as total distribution volume of [^{11}C]PBR28 in brain and in blood cells.

TSPO binding in brain was strongly and positively correlated to binding in blood cells both at baseline and when analyzing change between two PET examinations. Furthermore, there was a significant correlation between change of leukocyte numbers and change in TSPO binding in brain, and a trend-level correlation to change in TSPO binding in blood cells. These *in vivo* findings indicate an association between immunological cells in blood and brain *via* intact BBB, suggesting a functional interaction between these two compartments, such as interchange of peripherally derived cells or a common regulatory mechanism. Measurement of radioligand binding in blood cells may be a way to control for peripheral immune function in PET studies using TSPO as a marker of brain immune activation.

Abbreviations

AUC, area under the curve;

BBB, blood brain barrier;

HAB, high affinity binder;

HCT, hematocrit value;

LAB, low affinity binder;

MAB, mixed affinity binder;

PET, positron emission tomography;

ROI, region of interest;

TAC, time activity curve;

TSPO, 18-kDa translocator protein;

V_T, distribution volume;

Introduction

The brain was long considered to be an immunologically privileged organ due to the blood brain barrier (BBB) preventing entry of immune cells from the peripheral circulation. However, during the last decades it has become clear that circulating blood monocytes, progenitors of macrophages, can move into the brain through the intact BBB (Lawson et al., 1992; Persidsky et al., 1997; Fiala et al., 1997). Furthermore, in addition to parenchymal microglia the brain hosts also other myeloid populations such as macrophages in the choroid plexus, perivascular space and meninges (Prinz & Priller., 2014). Very recently, a lymphatic system has been identified in the dural sinuses, further suggesting transfer of immune cells between periphery and the central nervous system (Louveau et

al., 2015). Although brain microglia and peripheral macrophages have similar morphology and

phagocytic functions, microglia are thought to have distinct ontogenesis and kinetic features. In

contrast to peripherally derived cells, microglia are long-lived and are considered to be maintained

primarily by local self-renewal mechanisms (Ginhoux et al., 2010; Prinz & Priller, 2014). Importantly,

most reports of interactions between central and peripheral cell populations are based on preclinical

or *in vitro* studies. Whether blood monocytes can reach the human brain through intact BBB at

physiological conditions, or if there is an indirect link between immune cells in brain and blood in

humans is still largely unknown.

The translocator protein (TSPO), formally named the peripheral benzodiazepine receptor, is a

mitochondrial protein highly expressed in brain microglia and macrophages, and to some extent

astrocytes (Papadopoulos et al., 2006; Zavala et al., 1984; Venneti et al., 2013). In blood, the protein

is also expressed in monocytes and polymorphonuclear neutrophils, as well as to a lesser extent in

other cell types (Canat et al., 1993). Quantitative imaging of TSPO with positron emission

tomography (PET) may provide a biomarker for the activity of brain immune cells, and several TSPO

radioligands have accordingly been developed during recent years. Among those, the second

generation TSPO radioligand [^{11}C]PBR28 shows an increased signal-to-noise ratio compared to the

first-generation TSPO radioligand [^{11}C]PK11195 (Brown et al., 2007; Fujita et al., 2008; Imaizumi et al.,

2008; Kreisl et al., 2010; Jučaite et al., 2012) and has been used to demonstrate changes in the brain

of patients with inflammation-related diseases (Oh et al., 2011; Kreisl et al., 2013a).

There are three different TSPO binding phenotypes in human subjects, i.e. high, mixed and low-

affinity binders (HAB, MAB and LAB, respectively), for which the difference in binding affinity is

caused by a polymorphism of the *Tspo* gene (rs6971) leading to an amino-acid substitution

(Ala147Thr) (Owen et al., 2011, 2012). Since TSPO is expressed also in peripheral immune cells,

binding to this protein also outside of the brain might be affected by the *Tspo* polymorphism.

1   However, thus far there are no published quantitative studies on TSPO binding in peripheral blood

2   cells *in vivo*, and its relationship to TSPO binding in brain has not been explored.

3   As is the case for other TSPO radioligands, there is a high degree of inter-individual variability in

4   [11C]PBR28 binding, also after taking TSPO genotype into account (Fujita et al., 2008; Kreisl et al.,

5   2010; Narendran et al.,2014; Collste et al., 2015). Furthermore, we have recently reported a sizeable

6   test-retest variability of 18.2% in [11C]PBR28 binding in healthy control subjects (Collste et al., 2015).

7   In that study, a diurnal effect was observed for several measures where [11C]PBR28 binding

8   measured in the afternoon was higher compared to that in the morning, suggesting that part of the

9   variability may have a biological source. This is in line with experimental evidence showing that the

10   monocyte/microglia system is highly dynamic, as demonstrated for instance in studies showing rapid

11   recovery after experimental microglia depletion (Elmore et al., 2014). If there is a link between the

12   peripheral and central immune cells, such that changes in the peripheral immune system may

13   influence brain TSPO levels, this relationship could contribute to the intra- and inter-individual

14   variability of [11C]PBR28 binding in brain. In that case, controlling for change of peripheral TSPO

15   binding in brain PET measurements may allow for a more sensitive detection of brain-specific

16   immune activation both in case-control and longitudinal studies.

17   The primary aim of this study was to examine the association between TSPO-binding in peripheral

18   blood cells and brain. A secondary aim was to investigate whether accounting for TSPO binding in

19   blood could reduce variability in measurements of TSPO levels in brain. For that purpose, we

20   analysed [11C]PBR28 PET data from 32 healthy individuals, of which 26 underwent repeated

21   measurements. We calculated the distribution volume of [11C]PBR28 binding in blood cells ($V_{T\ Blood}$

22   $_{cells}$) from blood and plasma radioactivity and compared that to brain TSPO binding expressed as V_T

23   $_{Brain}$. Based on preclinical and *in vitro* experiments, we hypothesized that there would be an

24   association between these two parameters. Assuming that TSPO levels and blood immune cell

25   numbers may change also during physiological conditions, we furthermore compared the change of

1   $V_{T\ Brain}$ and $V_{T\ Blood\ cells}$ between two PET measurements, as well as the correlation between [^{11}C]PBR28

2   binding and blood leukocyte numbers.

3

1

2   **Materials and methods**

3   *Subjects*

4   This study was approved by the Regional Ethical Review Board in Stockholm, Sweden, and the

5   Radiation Safety Committee at Karolinska University Hospital, Stockholm, and was performed in

6   accordance with the current amendment of the Declaration of Helsinki and International Conference

7   on Harmonization/Good Clinical Practice guidelines. Two cohorts of healthy subjects were included

8   in this study (Table 1). Cohort 1 included 7 males and 6 females aged 24.1 $\pm$ 3.0 (mean $\pm$ SD), and

9   cohort 2 consisted of 9 males and 10 females aged 42.2 $\pm$ 13.4. All subjects were genotyped for the

10   rs6971 polymorphism using a Taqman SNP genotyping assay and classified as HAB, MAB and LAB

11   (n=18, 13 and 1, respectively).

12

13   *Table 1. Study design*

	Age (years)	Number (Gender M/F)				Intervals between two PET	Blood cell count
		Total	HAB	MAB	LAB		
Cohort 1[*1]	24.1 $\pm$ 3.0	13 (7/6)	6 (4/2)	6 (2/4)	1 (0/1)	the same day (3.4 $\pm$ 1.0 hr, n=6)	No
						separate days (4.0 ± 2.1 days, n=7)	
Cohort 2[*2]	42.2 $\pm$ 13.4	19 (9/10)	12 (6/6)	7 (3/4)	0	One measurement (n=6)	Yes[*3]
						separate days (158 ± 73 days, n=13)	

*1 All individuals underwent two PET measurements.
*2 Six individuals (4 HABs, 2 MABs) underwent only one PET measurement and the other 13 individuals underwent two measurements.
*3 In one individual (HAB), blood cell counts was not measured at PET2.
HAB = High affinity binders; MAB = Mixed affinity binders ; LAB = Low affinity binders.

14

15   *PET and MR measurements*

16   For 26 subjects (13 from cohort 1 and 2, respectively. See table 1), two PET measurements were

17   performed either on the same day (n=6, 3 HABs and 3 MABs, average time interval of 202 ± 62 min

18   between injections), in separate days with short intervals (n=7, 3 HABs, 3 MABs and 1 LAB, average

7

1   interval 4.0 ± 2.1 days), or in separate days with long intervals (n=13, 8 HABs and 5 MABs, average

2   interval 158 ± 73 days). Examinations were performed using the High Resolution Research

3   Tomograph (HRRT, Siemens Molecular Imaging, Knoxville, TN, USA). For each subject, a plaster

4   helmet was made that fixated the subject's head to the PET system in order to prevent head motion

5   during the PET examinations. A transmission scan was performed using a rotating ^{137}Cs source in

6   order to correct the emission data for signal attenuation.

7   [^{11}C]PBR28 was prepared as described previously (Collste et al., 2015) and was administered

8   intravenously as a bolus injection. Mean injected radioactivity in PET 1 for all subjects (n=32) was

9   408 ± 52 MBq, for PET 2 (n=26) 404 ± 42 MBq. Mean injected mass in PET 1 (n=32) was 0.52 ± 0.26

10   µg, in PET 2 (n=26) 0.61 ± 28 µg. The differences in injected radioactivity and specific activity for

11   those subjects that underwent two PET measurements were not statistically significant. PET images

12   were acquired in list mode for 93 minutes except for two individuals where data acquisition was

13   limited to 60 minutes for PET2 in cohort 1 (HAB, different day with short intervals), and 50 minutes

14   for PET1 in cohort 2 (HAB, different day with long intervals). For all subjects, the radioactivity level in

15   arterial blood (C_{Blood}) was measured *via* an automatic blood sampling system (ABSS; Allogg

16   Technology, Mariefred, Sweden) for the first 5 min, followed by manual sampling. Blood sampling

17   time was at 0 to 5, 10.5, 20, 30, 40, 50, 60, 70, 80, and 90 minutes in cohort 1, and at 0 to 5, 6, 8, 10,

18   15, 20, 25, 30, 40, 50, 60, 70, and 90 minutes in cohort 2. Radioactivity level in arterial plasma

19   (C_{Plasma}) was measured after centrifugation of the blood sample. To calculate plasma radioactivity for

20   the first 5 minutes, plasma measurements from manual blood samples (at 1 and 3 minutes for cohort

21   1, and 2 and 4 minutes for cohort 2) were linearly interpolated to create a time curve for plasma-to-

22   blood ratio, which was then multiplied with the blood curve from the automated sampling (Collste et

23   al., 2015). The parent fraction was determined by high-performance liquid chromatography as

24   described previously (Nakao et al., 2013). Metabolite analysis was performed at 1, 3, 5, 10.5, 20, 40,

25   60, and 90 minutes in cohort 1, and at 4, 10, 20, 30, 40, 50, 70, and 90 minutes in cohort 2.

1   PET images were reconstructed using the ordinary poisson ordered subset expectation maximization

2   algorithm, with 10 subsets and 16 iterations, including modeling of the system resolution in the

3   reconstruction process (Varrone et al., 2009). The reconstructed PET images were corrected for head

4   motion using a post-reconstruction frame-by-frame realignment process, as previously described

5   (Schain et al., 2012).

6   T1 weighted MR images were obtained for each individual, using the 1.5 Tesla using GE signa system

7   scanner (GE Medical Systems, Milwaukee, WI, USA), for coregistration with PET and definition of

8   Regions of Interest.

9   *Quantification of TSPO binding*

10   Regions of interest (ROIs) were obtained using the Automated Anatomical Labelling template (AAL)

11   using non-linear spatial normalization of MR images. The MR images and the ROIs were coregistered

12   to PET space using the rigid body transformation, and projected onto the dynamic PET image to

13   derive time activity curves (TACs). The normalization and coregistration procedures were performed

14   using SPM5 (Wellcome Department of Imaging Neuroscience at University College London). TSPO is

15   expressed throughout the brain, and since there was no hypothesis of regional differences in the

16   relationship to binding in blood cells, all AAL ROIs were combined into one large whole brain (WB)

17   ROI.

18   In order to allow for estimation of V_T values also in the LAB individual, where very low specific

19   binding is expected, the Logan graphical analysis approach was selected (Logan et al., 1990). $V_{T\,Brain}$

20   was calculated using the whole brain TAC and metabolite corrected plasma activity curve of parent

21   (input function) by fixing t^* to 30 minutes. All calculations of V_T were performed with PMOD v.3.2

22   and 3.3 (PMOD Technologies Ltd, Switzerland). For calculation of $V_{T\,Blood\,cells}$, the radioactivity in

23   blood cells in each time point was calculated from blood and plasma radioactivity and hematocrit

24   value (HCT) according to equation (1) (Fig. 1A), generating a TAC for blood cells. $V_{T\,Blood\,cells}$ was then

1   approximated as the ratio of $AUC_{Blood\ cells}$ and AUC_{input} of the entire scan time. Importantly, the blood

2   cell compartment is theoretically independent of the brain compartment (Fig. 1B).

3   $$C_{Blood\ cells} = \frac{\left[C_{Blood} - C_{Plasma} \cdot (1 - HCT)\right]}{HCT}$$ (1)

4

5   HCT values on the day of PET measurement were used in cohort 2. In cohort 1, HCT values were

6   obtained at screening visits before PET measurements. To validate this approach, we calculated the

7   intra-individual variability of HCT values in cohort 2 to be within 3%. For 14 individuals without HCT

8   values (9 HAB, 5 MAB (of which 5 HAB and 3 MAB had repeat measurements)), the population

9   average value of 0.429 was used (Jones et al., 2004). According to the hypothesis that TSPO binding

10 between brain and blood are correlated, normalized V_T in brain was calculated by dividing $V_{T\ Brain}$ with

11 $V_{T\ Blood\ cells}$
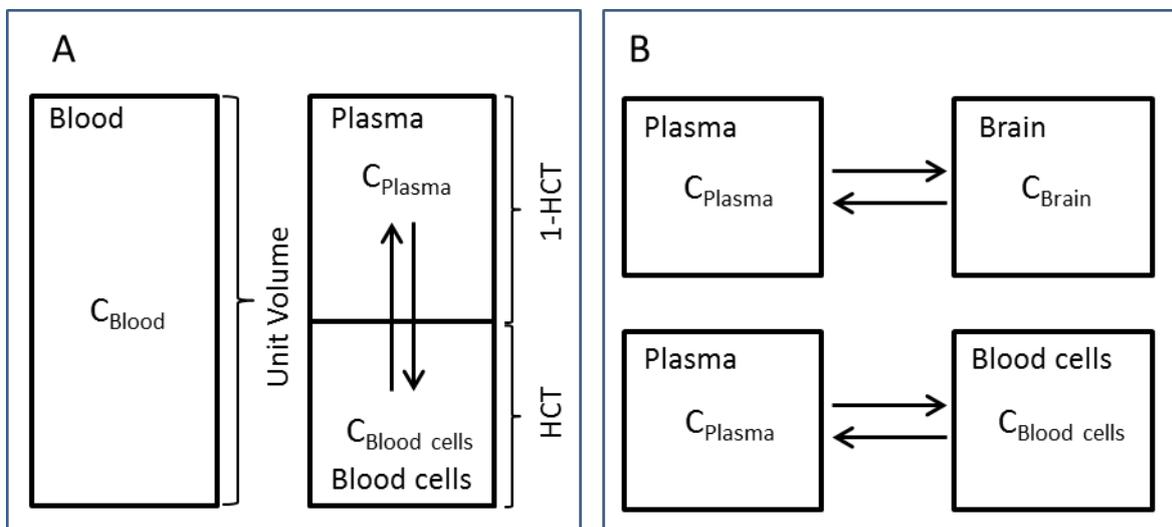
12

13 *Fig. 1. Mass balance between blood cells, brain and plasma compartments.* *(A) Mass balance in blood (left)*

14 *and plasma and blood cells (right). Assuming unit volume of blood, volume of plasma and blood cells are*

15 *represented using hematocrit values (HCT) as 1-HCT and HCT, respectively. The concentration of radioligand in*

16 *blood cells was calculated from the amount in blood cells (C_{Blood} x 1-C_{Plasma} x (1-HCT)) divided by volume of blood*

17 *cells (HCT). (B) Mass balance between plasma and brain (upper), and between plasma and blood cells*

18 *compartment (lower). V_T can be estimated by the ratio of concentration between tissue and plasma at*

1   *equilibrium. Assuming that radioactivity corresponds to the parent form of PBR28, at this state $V_{T\,Brain}$ and V_T*

2   *$_{Blood\,cells}$ are equal to to C_{Brain}/C_{Plasma} and $C_{Blood\,cells}/C_{Plasma}$, respectively.*

3

4   *Blood cells counts*

5   For 19 individuals from cohort 2, leukocyte counts were obtained in conjunction with PET

6   measurements. Briefly, venous blood were collected in ethylenediaminetetraacetic acid (EDTA)-

7   containing vacutainers *via* routine procedures for analysis of complete blood counts with leukocyte

8   differentials at the Karolinska University Hospital Laboratory, Solna, Sweden. Repeat measurements

9   were performed for 12 subjects (long interval).

10

11   *Statistics*

12   Coefficient of variance (COV) was calculated as the ratio of the standard deviation to the mean. The

13   change of brain and blood cell distribution volume (ΔV_T) was calculated according to equation (2).

14   Change for blood cell parameters (ΔN) as well as for normalized distribution volume in brain ($\Delta(V_T$

15     $_{Brain}/V_{T\,Blood\,cells}$)) were calculated in the same way. In a secondary analysis of specific cell types,

16   correction for multiple comparisons was applied.

17
$$\Delta V_T = \frac{V_T^{PET1} - V_T^{PET2}}{\frac{1}{2}\left(V_T^{PET1} + V_T^{PET2}\right)} \cdot 100 \qquad (2)$$

18   For the purpose of the investigation whether accounting for TSPO binding in blood could reduce

19   variability in measurements of TSPO levels in brain, absolute value of ΔV_T was used for the

20   calculation of absolute variability of two measurements. Group comparisons of mean V_T values

21   between HABs and MABs were calculated using Student's t-test. Since genotype has a major impact

22   on [11C]PBR28 V_T values this factor was controlled for in the analysis of associations between V_T

1   values in brain and blood cells, using a partial correlation (Kreisl et al., 2013a, Kenk et al., 2015, Lyoo

2   et al., 2015). Separate analyses were then performed for each genotype group using Pearson's

3   correlations. Partial correlations were performed also for analyses of V_T values in relation to blood

4   cell counts. For the association between change in V_T values and leukocyte counts, a secondary

5   analysis was performed for each genetic subgroup, using Spearman's correlation to avoid strong

6   effects from any single measurement in these very small sample sizes (n=7 for HAB and n=5 for

7   MAB). In contrast, genotype has shown not to affect variability of TSPO binding in brain (Collste et al.,

8   2015) and since we observed that binding class affects $V_{T\,Blood\,cells}$ to a similar extent, the main

9   analyses of correlations between change in V_T between brain and blood cells were performed for all

10   subjects combined using Pearson's correlations. To exclude a confounding effect of genotype, a

11   partial correlation was performed as a secondary analysis. For a separate analysis in individuals

12   examined during the same day (n=6) or on separate days within a week (n=6) correlations were

13   performed using Spearman's correlation. The significance level for all tests was set to 0.05.

14   *Estimation of [^{11}C]PBR28 binding amount in tissue*

15   Total [^{11}C]PBR28 binding amount in brain and blood cells in each genotype was estimated by

16   multiplying tissue actual volume and concentration ratio between tissue and plasma. Since

17   [^{11}C]PBR28 V_T to a significant extent is constituted by specific binding (Owen et al., 2014), these

18   values can be considered to reflect relative TSPO expression among tissues in each genetic group.

19

2 **Results**

3 [^{11}C]PBR28 Binding in Brain and Blood cells.

4 As expected, HAB, MAB and LAB individuals showed high, middle and low [^{11}C]PBR28 binding in brain

5 (Table 2). In PET1, the average value of $V_{T\,Brain}$ in MAB was 67 % of that in HAB subjects (p=0.007).

6 Similarly, HAB, MAB and LAB had high, middle and low binding in blood cells and the average value of

7 $V_{T\,Blood\,cells}$ in MAB was 76 % compared to that in HAB, a difference which was at trend-level

8 significance (p=0.066). $V_{T\,Brain}$ and $V_{T\,Blood\,cells}$ in LAB were less than 1, indicating no evident specific

9 binding of [^{11}C]PBR28 in any of the tissues. This individual was therefore excluded from the ensuing

10 analyses. There was a strong statistically significant correlation between $V_{T\,Brain}$ and $V_{T\,Blood\,cells}$ for HAB

11 and MAB subject for both PET1 (n=31, r=0.85, p=2.1 x 10^{-9}) (Fig. 2) and PET2 (n=25, r=0.90, p=3.7 x

12 10^{-9}). The correlations were also highly significant for each genetic group separately (Supplementary

13 table 1).

14 *Table 2. Mean distribution volume of [^{11}C]PBR28 in brain and blood cells*

Cohort 1+2

Distribution volume	PET1 (all subjects)		
	HAB (*n* = 18)	MAB (*n* = 13)	AB (*n* = 1)
$V_{T\,Brain}$ (mL·cm^{-3})	3.74 ± 1.30	2.51 ± 0.94	0.92
$V_{T\,Blood\,cells}$ (mL·mL^{-1})	1.72 ± 0.69	1.31 ± 0.37	0.71
$V_{T\,Brain}$/$V_{T\,Blood\,cells}$	2.26 ± 0.50	1.90 ± 0.43	1.29

Distribution volume	PET1 of repeat measurements			PET2 of repeat measurements		
	HAB (*n* = 14)	MAB (*n* = 11)	AB (*n* = 1)	HAB (*n* = 14)	MAB (*n* = 11)	LAB (*n* = 1)
$V_{T\,Brain}$ (mL·cm^{-3})	3.91 ± 1.40	2.45 ± 0.99	0.92	3.99 ± 1.54	2.70 ± 0.83	0.64
$V_{T\,Blood\,cells}$ (mL·mL^{-1})	1.80 ± 0.75	1.30 ± 0.40	0.71	2.03 ± 0.77	1.38 ± 0.68	0.43
$V_{T\,Brain}$/$V_{T\,Blood\,cells}$	2.27 ± 0.56	1.88 ± 0.45	1.29	1.96 ± 0.29	2.19 ± 0.71	1.49

Values are represented as mean ± SD.

15 HAB = High affinity binders; MAB = Mixed affinity binders ; LAB = Low affinity binders.
16

17

18

Plot annotation: $n = 31$, $r = 0.85$, $p = 2.1 \times 10^{-9}$

Y-axis: Unstandrdized residuals of $V_{T\,Brain}$

X-axis: Unstandardized residuals of $V_{T\,Blood\,cells}$
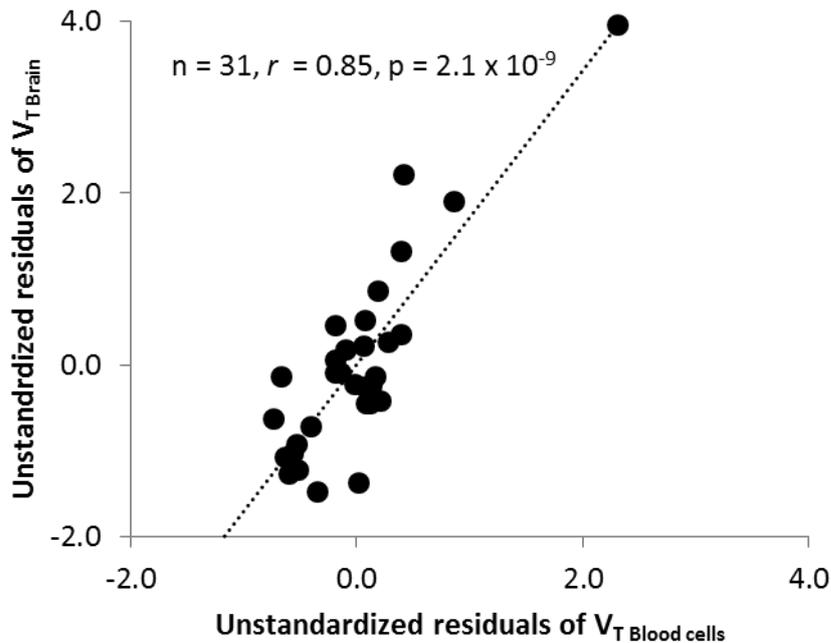
1

2   *Fig. 2. Partial correlation plot of [^{11}C]PBR28 binding in brain ($V_{T\,Brain}$) and blood cells ($V_{T\,Blood\,cells}$) at PET1, with*

3   *unstandardised residuals after accounting for genotype*

4

5   *Correlation between Change of [^{11}C]PBR28 Binding in Brain and Blood Cells.*

6   When analyzing repeat measurements, a statistically significant positive correlation was obtained

7   between changes in brain ($\Delta\,V_{T\,Brain}$) and blood cell binding ($\Delta\,V_{T\,Blood\,cells}$) (n = 25, r = 0.60, p=0.002)

8   (Fig. 3). The results remained at a high level of significance when performing a partial correlation,

9   indicating that the association was not caused by an effect of genotype (r = 0.67, p = 0.0003)

10 (Supplementary Fig. 2). In HAB and MAB, the changes in average AUC in brain, blood cells, and

11 plasma input from PET1 to PET2 were -5.8%, -3.3% and -11%, respectively (Supplementary table 2).

12 There was no change in parent fraction in plasma between PET measurements (Supplementary Fig.

13 1). When dividing subjects according to the time interval between examinations, significant

14 correlations were observed both for subjects examined in the same day or on separate days with a

15 short interval (n = 12, r = 0.85, p =0.001), whereas only a trend-level significance was obtained for the

16 group with a long interval between PET examinations (n = 13, r = 0.55, p = 0.054). For the short

1   interval group, the association appeared to be stronger for the same-day interval individuals

2   compared to the separate-day group (n=6, r = 0.94, p = 0.005 compared to n= 6, r = 0.77, p = 0.07).

3   When dividing the sample according to genotype, an association between $V_{T\,Brain}$ and $V_{T\,Blood\,cells}$ was

4   shown for HAB subjects only (n = 14, r=0.83, p=0.0002) (Supplementary Fig. 2).

5

6

7

8   **Fig. 3. Fig. 3. Correlation between change of [^{11}C]PBR28 binding in brain (Δ $V_{T\,Brain}$) and blood cells (Δ $V_{T\,Blood}$**

9   **$_{cells}$) between two PET measurements, showing individuals with short (<1 week) and long time intervals**

10   **between examinations.**
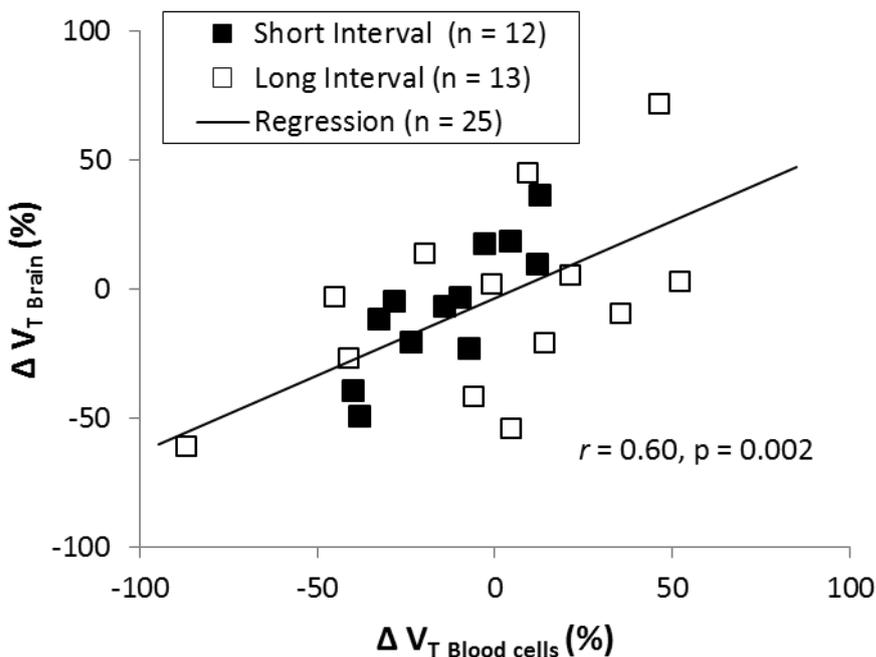
11

12   *Correlations between [^{11}C]PBR28 Binding in Brain and Blood Cells versus Blood Leukocyte Counts*

13   The leukocyte counts on the day of PET measurements are given in Table 3. When analyzing data for

14   PET1, a negative association was found between leukocyte numbers and $V_{T\,Brain}$ (n = 19, r = -0.49, p =

15   0.041) but not for $V_{T\,Blood\,cells}$ (n = 18, r = -0.27, p = 0.3). No statistically significant associations were

15

1   found for PET 2 (n=12, *r*=0.046, p=0.98 for V_{TBrain} ; *r*= 0.074, p=0.83 for $V_{TBlood\ cells}$). When comparing

2   the change in leukocyte numbers and change in V_T values in brain for repeat PET measurements, a

3   significant correlation was observed (n=12, *r* = 0.63, p = 0.038, Fig. 4A). A trend-level significance was

4   found for the association between change in leukocyte numbers and change in V_T values in blood

5   cells (*r* = 0.60, p=0.052, Fig. 4B). Associations for each genetic subgroup are shown in Supplementary

6   table 3. Regarding specific blood cell types, no significant correlation was found between leukocyte

7   counts and $V_{T\ Blood\ cells}$ or $V_{T\ Brain}$, either for the analysis at PET1 and PET2 or when assessing change

8   between the measurements.

9

10   ***Table 3. Leukocyte counts in venous blood***

Cohort 2

Blood cell (10^9/L)	PET1 (all subjects)			
	HAB (*n* = 12)		MAB (*n* = 7)	
Leukocytes	5.54 ± 1.46		4.60 ± 0.65	
Neutrophil grannulo	2.83 ± 0.97		2.51 ± 0.40	
Lymphocytes	2.07 ± 0.70		1.53 ± 0.22	
Monocytes	0.45 ± 0.17		0.44 ± 0.08	

Blood cell (10^9/L)	PET1 of repeat measurements		PET2 of repeat measurements	
	HAB (*n* = 7)	MAB (*n* = 5)	HAB (*n* = 7)	MAB (*n* = 5)
Leukocytes	5.13 ± 1.71	4.70 ± 0.66	6.09 ± 1.90	4.40 ± 0.80
Neutrophil grannulo	2.63 ± 0.96	2.56 ± 0.43	3.56 ± 1.25	2.30 ± 0.54
Lymphocytes	1.91 ± 0.65	1.60 ± 0.14	1.89 ± 0.70	1.68 ± 0.29
Monocytes	0.44 ± 0.14	0.42 ± 0.08	0.46 ± 0.10	0.38 ± 0.11

Measurements were performed in conjunction with [^{11}C]PBR28 PET examinations.
Values are represented as mean ± SD.
11   HAB = High affinity binders; MAB = Mixed affinity binders ; LAB = Low affinity binders.
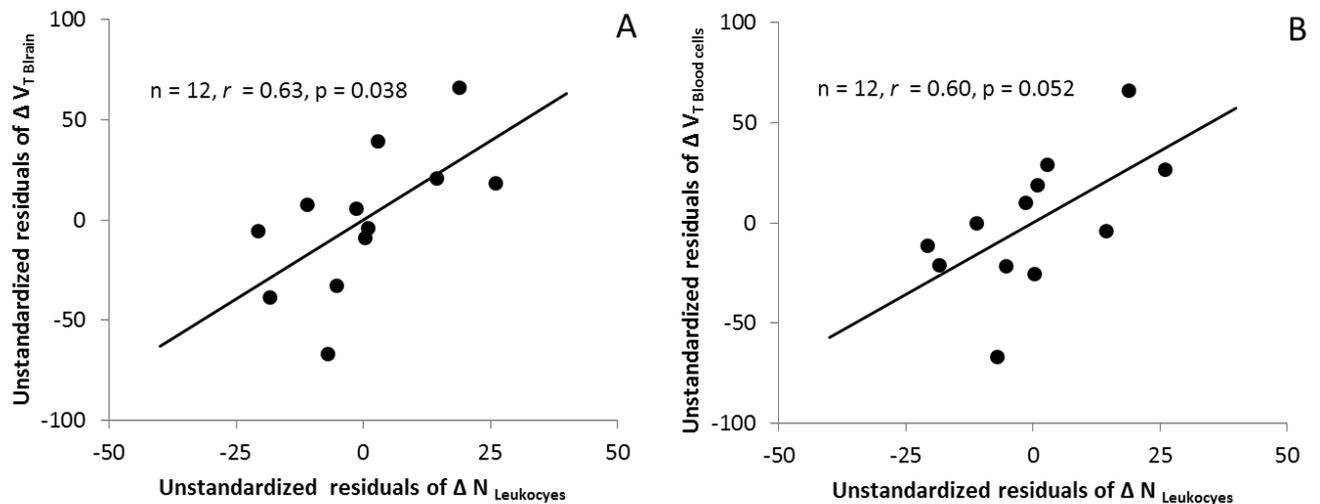
1

2

3 **Fig. 4. Partial correlation plots of percent change of leucocyte numbers and [^{11}C]PBR28 binding in brain (V_T**

4 **$_{Brain}$) (A) and blood cells ($V_{T\,Blood\,cells}$) (B) between two PET measurement, with unstandardised residuals after**

5 **accounting for genotype.**

6

7 *Normalization of [^{11}C]PBR28 Binding in Brain - effect on variability*

8 TSPO binding in brain was normalized to that in blood cells ($V_{T\,Brain}/V_{T\,Blood\,cells}$). The normalized values

9 were higher in HAB than in MAB in PET1 (p=0.045) (Table 2). For the whole sample as well as when

10 analyzing each genetic group separately, the inter-individual variability for PET1 was reduced when

11 compared to $V_{T\,Brain}$ (Table 1) (COV% 40.4 to 23.4 (HAB and MAB combined), 34.9 to 22.0 (HAB) and

12 37.5 to 22.4 (MAB)). When analyzing repeat measurements, the absolute variability of normalized V_T

13 $_{Brain}$ was 23.0 $\pm$ 15.9%, compared to 23.9 $\pm$ 20.3% for $V_{T\,Brain}$. For the subgroup of HABs and MABs

14 examined within a short time interval (n=12), the average absolute variability was reduced from 20.1

15 $\pm$ 14.7% for $V_{T\,Brain}$ to 12.4 $\pm$ 8.4% for normalized $V_{T\,Brain}$.

16 *Estimation of [^{11}C]PBR28 Binding Amount in Tissue*

17

1   To estimate the amount of TSPO in brain and blood cells respectively, total [11C]PBR28 binding was

2   calculated based on V_T values and approximate tissue volumes. Assuming that brain weight

3   constitutes 2% of body weight (20 mL/kg), the amount of [11C]PBR28 existing in brain calculated with

4   the ratio to plasma (i.e. V_T in each organ) was 75 mL/kg (=20 x 3.74) and 50 mL/kg (=20 x 2.51) in

5   HABs and MABs, respectively. Similarly, assuming a blood volume of 7% of body weight (70 mL/kg)

6   and hematocrit value of 0.45, the amount of [11C]PBR28 in blood cells was 54 mL/kg (=70 x 0.45 x

7   1.72) and 41 mL/kg (=70 x 0.45 x 1.31) in HAB and MAB, respectively.

8

9

1 **Discussion**

2 TSPO is expressed in cells participating in immune surveillance in blood and brain. Although

3 [^{3}H]PBR28 binding to TSPO in blood cells *in vitro* previously has been shown to correspond to TSPO

4 genotype, and hence also to [^{11}C]PBR28 binding in brain *in vivo* (Kreisl et al., 2013b), there are to our

5 knowledge no previous attempts to quantify TSPO in blood cells *in vivo* and compare to brain TSPO

6 levels. In this study, we applied the concept of distribution volume to blood cells to serve as a basis

7 for comparisons with TSPO binding in brain. We found that HAB, MAB and LAB status affected

8 binding not only in brain but also blood cells, supporting the view that [^{11}C]PBR28 binds specifically to

9 TSPO in blood cells. When analyzing the relationship between $V_{T\,Brain}$ and $V_{T\,Blood\,cells}$, a significant

10 correlation was obtained. Moreover, the intra-individual change in repeated PET measurements in

11 these independent compartments was positively correlated, and the change in leukocyte numbers

12 showed correlations to change $V_{T\,Brain}$ as well as a trend-level association for in $V_{T\,Blood\,cells}$. The

13 associations generally showed large effect sizes (r >0.5). Taken together, the results provide *in vivo*

14 evidence of an interplay between immune cells in brain and blood, representing a causal relationship

15 in either direction, or the existence of a common regulatory factor.

16 A large body of animal studies have demonstrated periphery-to-brain immune communication

17 (Dantzer et al., 2008). Specifically, exploring the effect of peripheral immune activation on brain

18 TSPO levels, systemic administration of E. coli lipopolysaccharide (LPS) to baboons has been shown to

19 lead to a 50% increase of [^{11}C]PBR28 binding in brain. Importantly, in that study post-mortem

20 immunohistochemistry showed that TSPO was co-localized with cells expressing the

21 microglial/macrophage marker CD68, while no signs of BBB disruption was observed (Hannestad et

22 al., 2012). Very recently, a similar study design was applied to healthy human subjects, whereby

23 intravenous injection of 1 ng/kg LPS resulted in a 30-60% increase in brain [^{11}C]PBR28 V_T values

24 (Sandiego et al., 2015). At a more general level, the clinical relevance of periphery-to-brain immune

25 interaction has been demonstrated by studies showing that systemic injection of LPS in healthy

1   humans was associated with a dysfunctional central pain modulation, indicating a cerebral influence

2   (Karshikoff et al., 2015) as well as studies from inflammatory diseases suggesting that peripheral

3   immune activation may affect activity and function of resident CNS cells (Inglis et al., 2007). Our

4   results of an association between TSPO levels in immune cells in the periphery and in the brain

5   suggest that the CNS immune system may be influenced by variations in the peripheral immune

6   system also at physiological conditions.

7   An important question is to which extent the interaction between the central and peripheral immune

8   systems occurs *via* direct entry of TSPO-expressing cells. Although parenchymal microglia are not

9   generally replaced by peripheral cells, other immune cells that may contribute to the TSPO signal in

10   brain include macrophages in perivascular space, the choroid plexus and meninges, that are thought

11   to be derived from haematopoetic cells (Prinz & Priller, 2014). Also, preclinical *in vitro* studies have

12   showed that circulating monocytes can move into brain through intact BBB. (Lawson et al., 1992)

13   (Persidsky et al., 1997; Fiala et al., 1997). In contrast, brain infiltration of neutrophils, which also

14   express TSPO, has only been observed following breakdown of BBB integrity (Miller, 1999). Our

15   estimations of total [^{11}C]PBR28 binding showed that the amount of TPSO existing in brain and blood

16   cells are of similar size. Thus, if change of binding in brain measured with PET in this study is driven

17   by an interchange of cells, this would require a high rate of passage of blood cells. The recent

18   observation of lymphatic vessels within the brain, which indicate a link between CNS and systemic

19   lymphoid systems, can be viewed as support for such mechanisms (Louveau et al., 2015) and it may

20   be speculated that this transfer largely consists of non-parenchymal cells. However, not all TPSO-

21   containing blood cells would be expected to take part in this exchange and consequently alternative

22   mechanisms such as immune signaling across the BBB may play a role. In the non-human primate

23   study by Hannestad and colleagues (Hannestad et al., 2012), there was a correlation between the

24   change in brain TPSO binding and peripheral inflammatory cytokine levels. Although the present

25   study was performed on healthy subjects, where only low level of activity of inflammatory cytokines

1 is expected, similar immune-to-brain pathways may be present also in the human brain at

2 physiological states.

3 Apart from a change in cell numbers, a further possibility is that the observed variation in [^{11}C]PBR28

4 binding was caused by alterations in TSPO levels in existing cells. Recently, it has been reported that

5 the PKCepsilon-ERK1/2-AP-1/STAT3 signal transduction pathway is a primary regulator of *Tspo* gene

6 expression in tissues expressing high levels of TSPO, and chemicals, hormones and environmental

7 factors may regulate the TSPO expression through this pathway at physiological conditions (Batarseh

8 et al., 2008, 2010, Batarseh, Papadopoulos, 2010). However, the time span of 6 to 24 hours during

9 which the primary regulation has been reported to occur *in vitro* cannot entirely explain the results

10 of the present study, indicating changes in TSPO binding in both brain and blood cells also in subjects

11 with less than 4 hours between PET examinations.

12 The innate immune system is highly adaptive to exogenous challenges, and peripheral immune cell

13 activity may also show considerable variation at physiological conditions (Haus et al., 1999).

14 Measuring TSPO binding in blood cells may offer a possibility to control for state of the peripheral

15 immune system in PET studies, allowing for more specific assessment of parenchymal brain cells such

16 as microglia. In the present study, the inter-individual variability for normalized brain V_T values (V_T

17 $_{Brain}$/$V_{T\,Blood\,cells}$) was lower when compared to $V_{T\,brain}$. With regard to the intra-individual variability, a

18 significant reduction using $V_{T\,Brain}$/$V_{T\,Blood\,cells}$ was observed only for individuals where the time

19 between examinations was short. One possible interpretation is that these short-term fluctuations

20 are in part determined by the peripheral immune system, propagating primarily into non-

21 parenchymal cells in the brain, whereas long-term variations may correspond to the slower biological

22 kinetic behavior of microglia. However, to what extent normalized values reflects the actual

23 physiological state of the *in situ* brain immune system has to be studied further.

24

25 *Limitations*

1   In this study, we applied the Logan plot for the calculation of $V_{T\,Brain}$ to all individuals in order to allow

2   for analysis also of the LAB subject, where a specific binding compartment theoretically should not

3   be present. This may affect the generalizability of our results to other quantitative approaches such

4   as the two tissue compartment model (2TCM). For instance, around 5% of the PET signal is thought

5   to originate from whole blood (i.e. blood cells and plasma), a factor which cannot be taken into

6   account using the Logan plot. However, linear approaches have been frequently used to quantify

7   [^{11}C]PBR28 binding (Hines et al., 2013, Varrone et al., 2013, Hannestad et al., 2013, Jucaite et al.,

8   2015, Park et al., 2015), and we recently reported a high level of correspondence between gray

9   matter binding as quantified using 2TCM and a parametric Logan approach (WAPI) (Collste et al.,

10   2015). For a subgroup of 13 HAB and MAB individuals in the present study, we compared WB V_T

11   calculated using Logan plot and 2TCM for PET1 and PET2 and confirmed that the values obtained for

12   the two methods were highly correlated (n= 26, r=0.979, p=1.35 x 10^{-16}).

13

14   An important limitation for the quantification of $V_{T\,Blood\,cells}$ is the potential influence of radioactive

15   metabolites in plasma at later time points. In contrast to brain, blood cells have no barrier to prevent

16   the entry of metabolites. Early after administration of the radioligand, the radioactivity in blood cells

17   would largely represent parent radioligand whereas the contribution of metabolites to binding in

18   blood cells likely is greater at later time points. If radioactive metabolites are present in blood cells,

19   this would lead to an overestimation of V_T. Nevertheless, since there are differences in blood to

20   plasma radioactivity ratio between binding phenotypes in absence of differences in plasma parent

21   fraction, blood cell binding is considered to reflect specific binding mainly. In addition, when we

22   calculated $V_{T\,Blood\,cells}$ by AUC ratio from 30 to 90 min, correlations between $V_{T\,Blood\,cells}$ and $V_{T\,Brain}$

23   remained at a high level of statistical significance (see supplemental Fig. 3).

24   Also, in the current study, we could not determine V_T for specific cell types, since the distribution

25   volume for blood cells was calculated using HCT which corresponds to the blood cell compartment as

1   a whole. However, this approach is supported by *in vitro* data suggesting that the major component

2   of TSPO radioligand binding in blood is accounted for by immune cells, whereas the contribution of

3   platelets or erythrocytes is less important in spite of their abundance in blood (Canat et al., 1993).

4   Based on B_{max} levels reported (Canat et al., 1993) and the cell counts in the present sample in

5   combination with population data, we estimated that >75% of the TSPO signal from blood cells

6   should be derived from leukocytes. However, further research is needed to clarify the relative

7   contribution of blood cell types to the demonstrated correlation between TSPO levels in blood cells

8   and brain.

9

Conclusions

We aimed to investigate the relationship between TSPO availability in brain and blood cells using PET and the radioligand [^{11}C]PBR28 in healthy human subjects. The results provide *in vivo* support of an association between immunological cells in blood and brain at physiological conditions. This relationship is of interest in relation to communication between the brain and peripheral immune systems and may constitute a specific source of inter- and intra-individual variability that could be controlled for in clinical PET studies using TSPO as a microglia biomarker.

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